

Raymond G. Goodwin\*,  
Wenjie S. Din\*,  
Terri Davis-Smith\*,  
Dirk M. Anderson\*,  
Steven D. Gimpel\*,  
Timothy A. Sato\*,  
Charles R. Maliszewski\*,  
Camillynn I. Brannan<sup>Δ</sup>,  
Neal G. Copeland<sup>Δ</sup>,  
Nancy A. Jenkins<sup>Δ</sup>,  
Terry Farrah\*,  
Richard J. Armitage\*,  
William C. Fanslow\* and  
Craig A. Smith\*

Immunex Research and  
Development Corporation\*, Seattle  
and ABL-Basic Research Program,  
NCI-Frederick Cancer Research  
and Development Center<sup>Δ</sup>,  
Frederick

## Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor\*

4-1BB is an inducible T cell antigen that shows sequence homology to members of an emerging family of cytokine receptors, including those for tumor necrosis factor and nerve growth factor. To aid in the analysis of the function of 4-1BB we have utilized a soluble form of the molecule as a probe to identify and clone the gene which encodes its ligand. The ligand for 4-1BB is a type II membrane glycoprotein that has homology to tumor necrosis factor, lymphotoxin, and the ligands for CD40 and CD27, all of which are themselves ligands to receptors in this superfamily. The gene for 4-1BB is on mouse chromosome 4 and maps close to the p80 form of the tumor necrosis factor receptor as well as the gene for CD30. The gene for 4-1BB ligand maps to mouse chromosome 17, but considerably distal to the tumor necrosis factor and lymphotoxin genes. Interaction of 4-1BB with its ligand induces the proliferation of activated thymocytes and splenic T cells, a response which is mimicked on similar cell populations stimulated with an antibody to 4-1BB.

### 1 Introduction

The cDNA termed 4-1BB was initially isolated by a differential screening procedure used to identify inducible, T cell-specific transcripts [1]. The predicted translation product contained a putative signal peptide and a transmembrane domain, raising the possibility 4-1BB might be a cell surface receptor for an unidentified ligand. Subsequently, sequence analysis of 4-1BB [2] showed it to be one member of an emerging superfamily of transmembrane proteins which include two receptors for tumor necrosis factor (TNF) [2-4], and a transcriptionally active open reading frame (T2) from Shope Fibroma Virus encoding a soluble homolog of the type II TNF receptor [2, 5]. In addition, this family includes the low-affinity nerve growth factor (NGF) receptor [6], the B cell surface antigen CD40 [7], two T cell activation antigens, OX40 [8] and CD27 [9], the surface antigen FAS, antibodies to which are capable of inducing apoptosis [10], and the lymphoid activation molecule CD30, which is expressed on the surface of the tumor cells of Hodgkin's disease [11].

While all of the members of the TNF/NGF receptor superfamily have characteristics of membrane-bound receptors, only ligands for the TNF and NGF receptors, and recently for CD40 [12] and CD27 [13], have been identified.

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\* Murine chromosomal mapping was supported in part by the National Cancer Institute, Department of Health and Human Services, under contract N01-CO-74101 with Advanced Bioscience Laboratory.

Correspondence: Raymond G. Goodwin, Immunex Research and Development Corporation, 51 University Street, Seattle, WA 98101, USA (Fax: 206 233-9733)

Abbreviations: NGF: Nerve growth factor LT: Lymphotoxin

Key words: Murine 4-1BB ligand / Type II protein

Because other members of this family also have characteristics of receptors (e.g., monoclonal antibodies directed against such putative receptors elicit cellular responses), and can be found on cells of the immune system, they constitute ideal candidates for use in a search for novel immunoregulatory cytokines.

Here we report the use of a soluble form of 4-1BB fused to the Fc region of human IgG1 as a probe to identify and clone its ligand. The 4-1BB ligand (4-1BB-L) is a type II membrane glycoprotein, with the carboxy-terminal domain extracellular. The extracellular domain of 4-1BB-L shows clear sequence homology to the corresponding regions of TNF, LT- $\alpha$  (lymphotoxin), LT- $\beta$  [14] and the ligands for CD40 (CD40L) and CD27 (CD27L), suggesting the emergence of a corresponding family of ligands parallel to the superfamily of receptors with which they interact.

### 2 Materials and methods

#### 2.1 Construction, expression and purification of a 4-1BB/Fc fusion molecule

PCR amplification was performed to obtain the extracellular domain of murine 4-1BB. The 5' oligonucleotide used in this reaction spanned nucleotides 70 to 90 of the published sequence [1] and was preceded by an Spe I cleavage site. The 3' antisense oligonucleotide spanned nucleotides 529 to 510 and was preceded by a Bgl II cleavage site. This allowed for its subsequent ligation to a human IgG1 sequence while maintaining the correct translational reading frame. The PCR reaction was performed using a cDNA template synthesized from RNA derived from an alloreactive T cell clone (BD14-20) which had been induced with Con A. Following amplification, the PCR product was digested with Spe I and Bgl II and gel-purified. The human IgG1 sequence was obtained by PCR amplification and subcloned into Bluescript SK (Stratagene). The 4-1BB and Fc (Bgl II and Spe I cut) fragments were then combined and

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 Interference No. 105,077  
 RX 2078

inserted into the mammalian expression vector pDC206 which is a derivative of pDC201 [15]. This vector was constructed such that sequences to be expressed are inserted into a unique Spe I site and are thus preceded by the murine IL-7 signal peptide [16]

Following construction of the 4-1BB/Fc fusion construct in pDC206, the entire insert including the murine IL-7 signal peptide was excised by digestion with Bam HI and the ends filled in with Klenow reagent. This fragment was then inserted into the expression vector pDC402 [17] at the Sal I site, the ends of which had also been blunted with Klenow reagent. This vector provides for improved expression of the fusion molecule for purification. Supernatants from CV-1/EBNA cells (ATCC CRL 104781; [17]) transfected with this plasmid were harvested after 7 days and passed through a protein G column. The column was then washed with phosphate-buffered saline (PBS) followed by elution of the bound 4-1BB/Fc fusion protein with 50 mM glycine, pH 3. The pH of the purified protein was then neutralized with 2 M Tris buffer, pH 9.

## 2.2 Radiolabeling and immunoprecipitations

Purified 4-1BB/Fc was radioiodinated to a specific activity of  $2 \times 10^5$  cpm/mmol with  $^{125}\text{I}$ , using a solid-phase procedure [2]. 4-1BB/Fc (10  $\mu\text{g}$ ), 1 mCi of neutralized  $\text{Na}^{125}\text{I}$  and 100  $\mu\text{l}$  of PBS were incubated in a 12  $\times$  75 mm glass tube previously coated with 5  $\mu\text{g}$  iodogen (Pierce, Rockford, IL) for 10 min at 4°C. The labeled protein was desalted on a 1 ml P6 (Biorad, Richmond, CA) gel filtration column, and stored at 4°C in binding medium (RPMI 1640, 0.1 M Hepes, 4% BSA, 0.02%  $\text{NaN}_3$ , pH 7) at a concentration of  $5 \times 10^{-8}$  M for up to 3 weeks with no detectable loss of activity.

Methods for  $^{35}\text{S}$  labeling of CV-1/EBNA cells transfected with either 4-1BB-L plasmid or with plasmid lacking an insert have been described [16]. Briefly, adherent cells in 10 cm plates, 2 days post-transfection, were washed twice with PBS, preincubated with  $\text{cys}^-/\text{met}^-$  RPMI 1640 (GIBCO, Gaithersburg, MD) for 30 min, then cultured for 4 h at 37°C in the same medium supplemented with 250  $\mu\text{Ci}/\text{ml}$  [ $^{35}\text{S}$ ]cysteine/[ $^{35}\text{S}$ ]methionine (1:1, ~1100 Ci/mmol each; Amersham, Arlington Heights, IL). Supernatants were removed, an aliquot mixed with equal volumes of 2  $\times$  SDS Laemmli buffer and subjected to SDS-PAGE on an 8-18% gradient gel. The gel was developed by phosphorimager (Molecular Devices, Palo Alto, CA). EL4 6.1 cells ( $2.5 \times 10^7$ ) were surface labeled with  $^{125}\text{I}$  using the lactoperoxidase procedure [18], then washed three times with PBS/0.15 M NaI. The resultant cell pellet was lysed with 3 volumes of 1% Triton X-100/PBS at 0°C for 20 min in the presence of a cocktail of protease inhibitors (1 mM PMSF, 1  $\mu\text{M}$  pepstatin A, 10  $\mu\text{M}$  leupeptin, 2 mM o-phenanthroline and 0.02 U/ml aprotinin), and subsequently microfuged for 20 min at 4°C to remove nuclei and cellular debris. Surface-labeling of transfected, adherent CV-1/EBNA cells was performed *in situ* (10 cm plates). These cells were lysed by addition of 1 ml PBS/1% Triton X-100 and processed as described above. Lysates were immunoprecipitated with protein G. Briefly, 0.5 ml of each lysate was precleared twice by incubation with 5  $\mu\text{g}/\text{ml}$  of human IgG1 and 50  $\mu\text{l}$  of a 20% Protein G suspension (Pharmacia, Piscataway,

NJ) for 2 h at 4°C, then microfuged for 2 min. The supernatants were then mixed with either IgG1 (control) or 4-1BB/Fc at 2  $\mu\text{g}/\text{ml}$  for 4 h at 4°C, then microfuged. Pellets were resuspended in 100  $\mu\text{l}$  of Laemmli SDS buffer, subjected to SDS-PAGE and visualized by phosphorimager.

## 2.3 Equilibrium binding studies

Equilibrium binding assays were performed as described [19]. For soluble, chimeric 4-1BB/Fc receptor binding to surface 4-1BB-L expressed on EL4 cells, serial dilutions of  $^{125}\text{I}$ -labeled 4-1BB/Fc ( $^{125}\text{I}$ -4-1BB/Fc) were incubated with  $2.5 \times 10^6$  EL4 cells in a total volume of 150  $\mu\text{l}$  of binding medium for 2 h at 4°C with constant mixing. For recombinant, surface 4-1BB-L expressed in COS-7 cells, the cells on 10 cm plates (used in transfections) were washed twice with PBS, then removed from the plates by treatment with 5 mM EDTA, pH 7.4, for 10 min at 37°C. Cells were washed once with binding medium and diluted 50-fold with carrier cells (RPMI 1788, previously shown to lack 4-1BB-L) to avoid clumping. This mixture was then added to serially diluted  $^{125}\text{I}$ -4-1BB/Fc. Following incubations, duplicate 60  $\mu\text{l}$  aliquots of the suspensions were microfuged for 2 min through plastic tubes containing a phthalate oil mixture [20] to separate bound and free  $^{125}\text{I}$ -4-1BB/Fc. Tubes were cut, and supernatant (free  $^{125}\text{I}$ -4-1BB/Fc) and cell pellets (bound  $^{125}\text{I}$ -4-1BB/Fc) were determined separately by gamma-counting. Nonspecific binding was determined by inclusion of a 200-fold molar excess of unlabeled 4-1BB/Fc. Binding curves were plotted in the Scatchard coordinate system.

Binding studies of soluble 4-1BB-L utilized supernatants from COS-7 cells transfected with a plasmid which expressed the soluble ligand. A soluble form of the 4-1BB-L was constructed in the pDC206 expression vector which utilizes the murine IL-7 signal peptide to promote secretion of the soluble 4-1BB-L product which encompasses amino acids 106 to 309. This construct was formed by ligating the 840 bp Asp 718/Bgl II fragment of the 4-1BB-L cDNA to Spe I/Bgl II cut pDC206 DNA in the presence of oligonucleotides to reconstruct the amino-terminal portion of the extracellular domain of 4-1BB-L and which contained overhanging sequences appropriate for ligation of the DNA.

## 2.4 Screening of the cDNA expression library

Subconfluent monolayers of COS-7 cells on fibronectin-treated chamber slides (Labtek) were transfected with 2  $\mu\text{g}$  of plasmid DNA from pools of 2000 clones using DEAE-dextran followed by chloroquine treatment [17]. The cDNA library, prepared from EL4 6.1 C10 RNA, utilized the mammalian expression vector pDC201 and has been previously described [15]. Three days after transfection, the slides were incubated with RPMI 1640 containing 25 mg/ml bovine serum albumin, 2 mg/ml sodium azide, 20 mM Hepes (pH 7.2), 50 mg/ml nonfat dry milk and  $1 \times 10^{-9}$  M  $^{125}\text{I}$ -4-1BB/Fc fusion protein. After 2 h at 4°C, the slides were washed three times, fixed with 2.5% glutaraldehyde in PBS then dried and dipped in liquid photographic emulsion as previously described [21]. After a 4 day exposure, the slides were developed and inspected microscopically to detect positive cells.

## 2.5 RNA blot analysis

Total cellular RNA was isolated by the guanidine isothiocyanate-cesium chloride method [22]. RNA was fractionated on 1.1% agarose-formaldehyde gels, blotted onto Hybond-N (Amersham) and UV-crosslinked using a Stratilinker (Stratagene). Filters were probed with a  $^{32}$ P-labeled antisense riboprobe as previously described [23]. As template for synthesis of the riboprobe we used pBluescript SK (Stratagene) containing the 840 bp Asp 718/Bgl II fragment of the 4-1BB-L cDNA.

## 2.6 Interspecific backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J  $\times$  *M. spretus*)F<sub>1</sub> females and C57BL/6J males as described [24]. A total of 205 N<sub>2</sub> progeny were obtained; a random subset of these N<sub>2</sub> mice were used to map the 4-1bb and 4-1bb-1 loci (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described [25]. All blots were prepared with Zetabind nylon membrane (AMF-Cuno). The probe for 4-1bb, a 680 bp Not I fragment, was labeled with [ $\alpha$ - $^{32}$ P]dCTP using a random prime labeling kit (Amersham); washing was done to a final stringency of 0.1  $\times$  SSCP, 0.1% SDS, 65°C. Fragments of 15.0 and 6.0 kb were detected in Bgl I-digested C57BL/6J DNA and fragments of 7.2, 6.6, 3.6, and 2.1 kb were detected in Bgl I-digested *M. spretus* DNA. All four *M. spretus*-specific Bgl I fragments cosegregated and their presence or absence was followed in backcross mice.

A description of the probes and RFLP for the loci linked to 4-1bb including Gardner-Rasheed feline sarcoma viral oncogene homolog (*Frg*), tumor necrosis factor receptor-1 (*Tnfr-1*) and Sloan-Kettering viral oncogene homolog (*Ski*) has been reported previously [26]. One locus used to position 4-1bb on the genetic map has not been reported previously for this backcross. The probe for the epithelial cell kinase (*Eck*) was a ~3 kb human cDNA from the catalytic domain [27]. At high stringency (final wash: 0.2  $\times$  SSCP, 0.1% SDS, 65°C), a 6.2 kb fragment was detected in Eco RI-digested C57BL/6J DNA and a 8.4 kb fragment was detected in Eco RI-digested *M. spretus* DNA. The presence or absence of the *M. spretus*-specific Eco RI fragment was followed in this analysis.

The probe for 4-1bb-1, a 1200 bp mouse full-length cDNA, was labeled by nick translation (Boehringer Mannheim); washing was done to final stringency of 0.1  $\times$  SSCP, 0.1% SDS, 65°C. Fragments of 7.4 and 0.9 kb were detected in Hinc II-digested C57BL/6J DNA and fragments of 4.6 and 0.9 kb were detected in Hinc II-digested *M. spretus* DNA. The inheritance of the 4.6 kb *M. spretus*-specific Hinc II fragment was followed in backcross mice. The probe and RFLP for the histocompatibility-2 (*H-2*) locus have been reported [28]. The probe for preferred integration site, moloney virus-2 (*Pim-2*), was a 1.5 kb mouse genomic DNA [29] that detected a 1.9 kb fragment in Msp I-digested C57BL/6J DNA and a 2.1 kb fragment in Msp I-digested *M. spretus* DNA. The *Pim-2* probe also detected a 9.6 kb fragment in Hind III-digested C57BL/6J DNA and a 4.6 kb fragment in Hind III-digested *M. spretus* DNA. The

probe for the *vav* proto-oncogene (*Vav*) was a ~2.6 kb mouse cDNA [30] that detected fragments of 13.5, 8.0, 6.4, 3.9, 3.5 and 2.3 kb in Bam HI-digested C57BL/6J DNA and 8.9, 6.4, 4.6, 3.9, 3.6, 2.4 and 1.0 kb fragments in Bam HI-digested *M. spretus* DNA. The 8.9, 4.6, and 1.0 kb Bam HI *M. spretus*-specific fragments cosegregated and were followed in the backcross. The probe for a *fasl*/fcs related nonreceptor protein-tyrosine kinase (*Fer*) was a ~950 bp rat cDNA [31] that detected 14.0, 3.5, 3.3, 2.8, 2.0, 1.4, and (1.1) kb fragments in Pvu II-digested C57BL/6J DNA and 14.0, 4.4, 3.8, 3.3, 2.6, (2.0), (1.4) and (1.1) kb in Pvu II-digested *M. spretus* DNA. The 4.4, 3.8 and 2.6 kb *M. spretus*-specific Pvu II fragments cosegregated and were followed in this study. Fragments shown in parentheses were light in intensity and not visible in all samples. The probe for laminin, A subunit (*Lama*) was a 1.15 kb mouse cDNA [32] that detected 5.8, 3.6, 3.0, 1.5, and 0.92 kb fragments in Taq I-digested C57BL/6J DNA and 4.2, 3.4, 2.6, and 1.4 kb fragments in Taq I-digested *M. spretus* DNA. The 4.2, 3.4, and 2.6 kb fragments cosegregated and were followed in this analysis. Recombination distances were calculated as described [33] using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allelic distribution patterns.

## 2.7 Proliferation assay

Assays studying the biological activity of the 4-1BB-L utilized CV1/EBNA cells which had been transfected with an expression vector (pDC402) containing the 4-1BB-L cDNA or with empty vector. Three days post-transfection the cells were fixed in 1% paraformaldehyde for 5 min at 4°C and these cells were diluted to various concentrations in assay medium.

Thymocytes from 8- to 12-week-old C67BL/6 mice (Charles River) were cultured in flat-bottom 96-well microtiter plates (3  $\times$  10<sup>5</sup> per well) in 200  $\mu$ l DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 10  $\mu$ M Hepes, 50  $\mu$ M 2-mercaptoethanol, 1 mM sodium pyruvate, 0.5 mM L-arginine, 0.3 mM L-asparagine, 14  $\mu$ M folic acid, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM glutamine. In addition, the cultures contained 1  $\mu$ g/ml concanavalin A (Sigma) and 1 ng/ml recombinant murine IL-2. After two days, wells were pulsed with 2  $\mu$ Ci [ $^3$ H]dThd (25 Ci/mmol) for 18 h, and then harvested onto glass fiber filters and cpm incorporation measured.

Purified naive T cells were obtained from the spleens of 8- to 12-week-old C57BL/6 mice. Spleen cell suspensions were prepared and then loaded onto anti-Ig columns (IsoCell, Pierce Chemical Co., Rockford, IL). The nonadherent cells were eluted from the anti-Ig columns according to the manufacturer's instructions. These eluted cells were examined by flow cytometry as described [34] using the CD3 mAb (500A2-FTTC conjugate, Pharmingen) and the goat F(ab')<sub>2</sub> anti-IgM ( $\mu$ )-FTTC conjugate (Tago, Burlingame, CA). Single-color flow cytometric analysis revealed that these cells were >93% CD3<sup>+</sup> and <1% surface IgM<sup>+</sup>.

For T cell functional assays, purified T cells were cultured for 3 days in 96-well round-bottom microtiter plates at a

density of  $1 \times 10^6$  cells/ml in a 200  $\mu$ l volume of medium. The culture medium was DMEM supplemented with 5% FBS,  $5 \times 10^{-5}$  M 2-ME and additional amino acids [35]. For proliferation assays, purified T cells were cultured in triplicate with a sub-optimal dose (0.05%) of Phytohemagglutinin (PHA-M; Difco) in the presence and absence of a varying concentration of fixed CV-1/EBNA cells expressing murine 4-1BB-L in a humidified atmosphere of 10% CO<sub>2</sub>. The cell-containing wells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]dThd (25 Ci/mmol) for the final 16 h of culture, cells harvested, and incorporated cpm determined by tritium-sensitive avalanche gas ionization detection on a Matrix 96 Direct Beta Counter (Packard, Meridew, CT).

## 2.8 Sequence analysis

Nucleotide and protein sequence analysis was performed using the Wisconsin Package [36]. Sequences were aligned using MALIGNED [37]. In nonconserved regions, a pattern-based turn prediction method [38] was used to align 4-1BB with TNF and LT- $\alpha$ .

## 3 Results

### 3.1 Isolation and expression of 4-1BB constructs

Our strategy for identification of the ligand for 4-1BB involved the construction of a soluble form of the putative receptor (encompassing amino acids 24-176; [1]) which deleted the transmembrane and cytoplasmic domains. This was performed by PCR utilizing as a template cDNA synthesized from RNA derived from a murine T cell clone which had been induced with Con A. In addition, we had previously shown that while a monomeric soluble form of the type II TNF receptor bound with low affinity, a dimeric Fc-TNF receptor fusion construct bound TNF with at least 50 $\times$  higher affinity [39], consistent with the oligomeric nature of the ligand. Thus, to ensure high-affinity binding of the soluble 4-1BB receptor to its putative ligand, and to aid in soluble receptor purification, we fused the receptor to the Fc portion of human IgG1. Similar receptor fusion constructs were used in the cloning of CD40L and CD27L. The 4-1BB/Fc fusion construct was inserted into a mammalian expression vector (pDC206) previously constructed such that the expressed protein would be preceded by the

AGCCTATAAAGCAGGGCACTGGCGGAGACGTGCACTGACCGACCGTGGA	Met Asp Gln His Thr Leu Asp Val Glu Asp Thr	11
	ATG GAC CAG CAC ACA CTT GAT GTG GAG GAT ACC	85
Ala Asp Ala Arg His Pro Ala Gly Thr Ser Cys Pro Ser Asp	Ala Ala Leu Leu Arg Asp Thr Gly Leu Leu Ala	36
GCG GAT GCC AGA CAT CCA GCA GGT ACT TCG TGC CCC TCG GAT	GCG GCG CTC CTC AGA GAT ACC GGG CTC CTC GCG	160
Asp Ala Ala Leu Leu Ser Asp Thr Val Arg Pro Thr Asn Ala	Ala Leu Pro Thr Asp Ala Ala Tyr Pro Ala Val	61
GAC GCT GCG CTC CTC TCA GAT ACT GTG CCG CCC ACA AAT GCG	GCG CTC CCC ACG GAT GCT GCG TAC CCT GCG GTT	235
Asn Val Arg Asp Arg Glu Ala Ala Trp Pro Pro Ala Leu Asn Phe	Cys Ser Arg His Pro Lys Leu Tyr Gly Leu	86
AAT GTT CCG GAT CCG GAG GCG TGG CCG CCT GCA CTG AAC TTC	TGT TCC CGC CAC CCA AAG CTC TAT GCG CTA	310
Val Ala Leu Val Leu Leu Leu Leu Ile Ala Ala Cys Val Pro Ile	Phe Thr Arg Thr Glu Pro Arg Pro Ala Leu	111
GTC GCT TTG GTT TTG CTG CTT CTG ATC GCG TGT OCT ATC TTC	ACC CGC ACC GAG GCG CCG CCA GCG CTC	385
Thr Ile Thr Thr Ser Pro Asn Leu Gly Thr Arg Glu Asn Asn Ala	Asp Gln Val Thr Pro Val Ser His Ile Gly	136
ACA ATC ACC ACC TCG CCC AAC CTG GGT ACC CGA GAG AAT AAT	GCA GAC CAG GTC ACC OCT GTT TCC CAC ATT GGC	460
Cys Pro Asn Thr Thr Gln Gln Gly Ser Pro Val Phe Ala Lys Leu	Leu Ala Lys Asn Gln Ala Ser Leu Cys Asn	161
TGC CCC AAC ACT ACA CAA CAG GCG TCT CCT GTG TTC GCC AAG	CTA CTG GCT AAA AAC CAA GCA TCG TTG TGC AAT	535
Thr Thr Leu Asn Trp His Ser Gln Asp Gly Ala Gly Ser Ser Tyr	Leu Ser Gln Gly Leu Arg Tyr Glu Glu Asp	186
ACA ACT CTG AAC TGG CAC AGC CAA GAT GGA GCT GGG AGC TCA	TAC TCT CAA GGT CTG AGG TAC GAA GAA GAC	610
Lys Lys Glu Leu Val Val Asp Ser Pro Gly Leu Tyr Tyr Val Phe	Leu Glu Leu Lys Leu Ser Pro Thr Phe Thr	211
AAA AAG GAG TTG GTG GTA GAC AGT CCC GGG CTC TAC TAC GTA	TTT TTG GAA CTG AAG CTC AGT CCA ACA TTC ACA	685
Asn Thr Gly His Lys Val Gln Gly Trp Val Ser Leu Val Leu Gln	Ala Lys Pro Gln Val Asp Phe Asp Asn	236
AAC ACA GGC CAC AAG GTG CAG GCG TGG GTC TCT CTT GTT TTG	CAA GCA AAG CCT CAG GTA GAT GAC TTT GAC AAC	760
Leu Ala Leu Thr Val Glu Leu Phe Pro Cys Ser Met Glu Asn Lys	Leu Val Asp Arg Ser Trp Ser Gln Leu Leu	261
TTG GCG CTG ACA GTG GAA CTG TTC CCT TGC TCC ATG GAG AAC	AAG TTA GTG GAC CGT TCC TGG AGT CAA CTG TTG	835
Leu Leu Lys Ala Gly His Arg Leu Ser Val Gly Leu Arg Ala Tyr	Leu His Gly Ala Gln Asp Ala Tyr Arg Asp	286
CTC CTG AAG GCT GCG CAC CGC CTC AGT GTG GGT CTG AGG GCT	TAT CTG CAT GGA GCC CAG GAT GCA TAC AGA GAC	910
Trp Glu Leu Ser Tyr Pro Asn Thr Thr Ser Phe Gly Leu Phe Leu	Val Lys Pro Asp Asn Pro Trp Glu End	309
TGG GAG CTG TCT TAT CCC AAC ACC AGC TTT GGA CTC TTT CTT	GTG AAA CCC GAC AAC CCA TGG GAA TGA GA	984
ACTATCCTCTCTGTGACTCTAGTGTGCTAAGTCTCAAGCTGCTATGTTT	TATGGGGTCTGAGCAGGGGTCCCTTCCATGACTTCTCTTGTCTTTAAC	1083
TGGACTTGGTATTATTTCTGAGCATAGCTCAGACAAGACTTTATATAAT	CACTAGATAGCATTAGTAAACTGCTGGCGAGCTGCTAGATAAAAAAAA	1182
TTTCTAAATCAAGTTTATATTTATATATATATAAAAAATAAATGTGTT	TGTAATAAAAA	1254

Figure 1. Nucleotide and predicted amino acid sequence of murine 4-1BB-L. The predicted transmembrane domain is boxed, and the polyadenylation signal is underlined. Asterisks indicate potential N-linked glycosylation sites.

murine IL-7 signal peptide [16] to ensure efficient secretion of the desired product.

While performing PCR reactions to isolate the extracellular domain of the 4-1BB receptor, we also performed reactions to isolate full-length molecules. In addition to the expected full-length PCR product, we observed a band migrating faster than the predicted product (data not shown). Cloning and sequencing of the PCR product revealed it lacked nucleotides 539 to 673 of the published sequence [1]. The predicted protein product of this clone lacks the transmembrane domain and, thus, presumably encodes an alternatively spliced, soluble form of 4-1BB.

### 3.2 Isolation of a cDNA encoding the ligand for 4-1BB

The purified 4-1BB/Fc fusion protein was used to screen for a cellular source of its cognate by two methods. First, the possible presence of a secreted, soluble ligand was examined by incubating 4-1BB/Fc with  $^{35}\text{S}$ -labeled cellular supernatants, precipitation of the complex with protein G, followed by SDS-PAGE and autoradiography. Alternatively, biotinylated or surface binding of  $^{125}\text{I}$ -4-1BB/Fc to a variety of cell types was employed to detect a surface form of the ligand. This latter procedure identified the murine thymoma cell line EL4 as a source of a membrane-bound ligand. We had previously constructed a cDNA expression library using RNA derived from a subclone of EL4 (EL4 6.1C10) which is enriched for the expression of the type I-IL-1 receptor [15]. Plasmid DNA from pools of roughly 2000 transformants of this library was isolated and transfected into COS-7 cells, which were then screened for their ability to bind radioiodinated 4-1BB/Fc using a microscopic autoradiographic approach [21]. After screening 200 pools, we obtained two pools positive for 4-1BB/Fc binding. The cDNA from both were subsequently isolated and their nucleotide sequences determined, one of which is shown in Fig. 1. Comparison of the 4-1BB-L sequence against the GenBank, EMBL, PIR-Protein and Swiss Prot computer databases revealed it is unique, though it does show homology to TNF, LT- $\alpha$ , LT- $\beta$ , CD40L, and CD27L (see Sect. 4). The sequence of the clone not shown is identical but lacks 34 nucleotides at the 5' end and contains one additional adenine residue at the 3' end. Both clones contain a single large open reading frame capable of encoding 309 amino acids. Hydrophilicity analysis predicts a single hydrophobic domain (amino acids 83 to 103). Conspicuously absent is a signal sequence, suggesting this protein is a type II membrane protein with its carboxy-terminal domain extracellular. This is consistent with the presence of three potential N-linked glycosylation sites present in the carboxy domain (Fig. 1), and has been confirmed by expression studies (see below). The extracellular region of the protein just downstream of the transmembrane domain may also contain O-linked carbohydrates as it is rich in threonine, serine and proline residues which are indicative of such modifications. The cDNA also contains a polyadenylation signal, underlined in Fig. 1.

### 3.3 Characteristics of native and recombinant 4-1BB ligand

The equilibrium receptor binding characteristics of native and recombinant surface 4-1BB-L are assessed in Fig. 2A

and B, respectively.  $^{125}\text{I}$ -labeled 4-1BB/Fc bound 4-1BB-L on EL4 cells with a single class of high-affinity sites ( $K_d = 0.5 \pm 0.05 \text{ nM}$ ;  $N = 1500 \pm 400$ ). The recombinant 4-1BB-L expressed on COS-7 cells bound the chimeric soluble receptor with an affinity very similar to the native form ( $K_d = 0.34 \pm 0.05 \text{ nM}$ ). Binding was saturable in both cases, and the affinity numerically similar to most cytokine-receptor interactions, including other known members of this emerging family (e.g., TNF, CD27L and CD40L); [2, 12, 13, 39]. The affinity measured here presumably reflects the intrinsic affinity of the surface receptor for the surface ligand on apposing cells, and is consistent with ligand-induced receptor cross-linking as the primary activation mechanism.

We also generated a construct which encoded a soluble form of 4-1BB-L, which lacks both N-terminal and transmembrane domains of the protein. The encoded protein encompassed amino acids 106-309 of 4-1BB-L and is preceded by the murine IL-7 signal peptide to promote secretion. Supernatant from cells transiently expressing soluble 4-1BB-L could completely inhibit  $^{125}\text{I}$ -4-1BB/Fc binding to surface 4-1BB-L (Fig. 2C). Control supernatants from cells transfected with vector lacking insert showed no inhibition.

The molecular weights and oligomeric status of the native and recombinant membrane-bound 4-1BB-L, as well as a recombinant soluble 4-1BB-L, were deduced from the

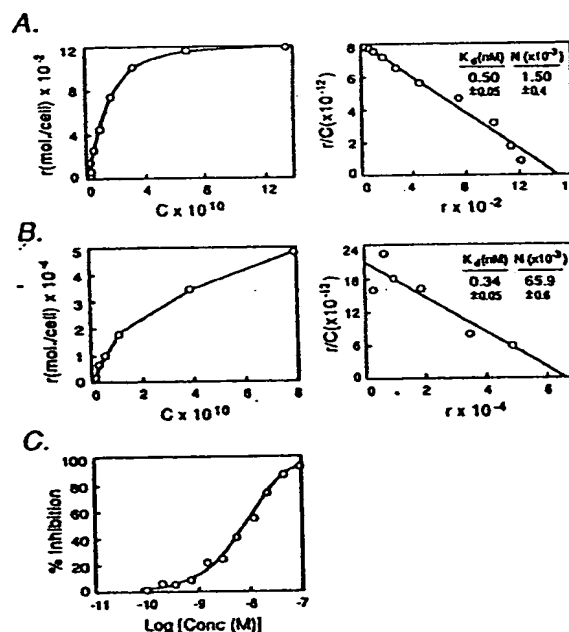
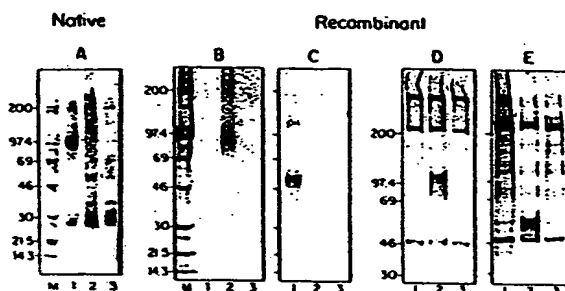


Figure 2. Equilibrium receptor binding characteristics of native (A) and recombinant (B) 4-1BB-L.  $^{125}\text{I}$ -4-1BB/Fc saturation isotherms at left replotted in Scatchard coordinates at right. The affinity of soluble receptor for both forms of surface ligand is nearly identical. (C) Inhibition of  $^{125}\text{I}$ -4-1BB/Fc binding to surface 4-1BB-L by COS-7 supernatants expressing soluble 4-1BB-L.



**Figure 3.** SDS-PAGE analysis of native and recombinant 4-1BB-L. EL4 6.1 cells expressing native ligand, COS-7 cells transiently expressing full-length, recombinant ligand (COS/4-1BB-L) and COS-7 cells transfected with vector lacking insert (COS/mock) were surface labeled with  $^{125}$ I. Detergent solubilized lysates were immunoprecipitated (see Sect. 2.2) with the following agents, and pellets resolved by SDS-PAGE under reducing (panels A, C and E) or nonreducing (panels B and D) conditions. The gel was developed by phosphorimager. A1: EL4 6.1 + M5 monoclonal antibody to the type I IL-1 receptor [61]; A2: EL4 6.1 + 4-1BB/Fc; A3: EL4 6.1 + IgG1; B1 and C2: COS/4-1BB-L + hulgG1; B2 and C1: COS/4-1BB-L + 4-1BB/Fc; B3 and C3: COS/mock + 4-1BB/Fc. COS-7 cells transiently expressing a secreted, soluble, recombinant 4-1BB-L and COS/mock cells were  $^{35}$ S-labeled and supernatants immunoprecipitated. D1 and E1: COS/mock + 4-1BB/Fc; D2 and E2: soluble 4-1BB-L + 4-1BB/Fc; D3 and E3: soluble 4-1BB-L + IgG1. Molecular weight markers are shown on the left.

SDS-PAGE analysis shown in Fig. 3. EL4 6.1 cells, COS-7 cells transiently expressing recombinant 4-1BB-L, or COS-7 cells transfected with vector lacking insert were surface labeled with  $^{125}$ I, and the cells lysed with PBS/1% Triton in the presence of protease inhibitors. Lysates were immunoprecipitated with 4-1BB/Fc and protein G, as well as controls, and the washed precipitates electrophoresed through an SDS gel under either reducing or nonreducing conditions. The apparent molecular mass of the native (Fig. 3A2) or recombinant (Fig. 3C1) 4-1BB-L under reducing conditions was ~50 kDa. The M5 monoclonal antibody (Fig. 3A1, positive control) to the type I IL-1 receptor could specifically immunoprecipitate the 97 kDa receptor from EL4 6.1 cells (Fig. 3A3), or COS-7 cells expressing recombinant 4-1BB-L (Fig. 3B1, 3C2). Similarly, 4-1BB/Fc did not immunoprecipitate any proteins from mock-transfected COS-7 cells (Fig. 3B3, 3C3). Since the calculated molecular weight of 4-1BB-L is 34 kDa, at least one of three potential N-linked sites, and perhaps the putative O-linked sites as well, are utilized. Under nonreducing conditions, however, recombinant 4-1BB-L appears at ~97 kDa (Fig. 3B2), suggesting the ligand is a disulfide-linked homodimer. Similar experiments were performed with metabolically labeled ( $^{35}$ S) COS-7 cells transiently expressing a secreted, soluble 4-1BB-L (C-terminal portion only). 4-1BB/Fc could immunoprecipitate an ~40 kDa protein from supernatants of cells transfected with vector containing the insert (Fig. 3D2, 3E2), but not from mock-transfected cells (Fig. 3D1, 3E1). IgG1 also did not bring down protein from supernatants of soluble 4-1BB-L transfectants (Fig. 3D3, 3E3). As with the full-length ligand, the soluble form under reducing conditions displays a molecular mass

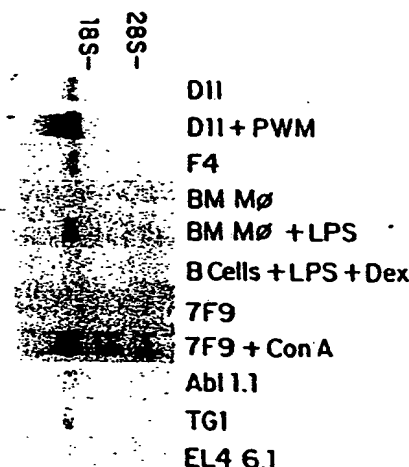
higher than predicted (35 vs. 21 kDa) and also runs at approximately twice this size under nonreducing conditions. Thus, both full-length and soluble forms of recombinant 4-1BB-L suggest the C-terminal 200 residues contain glycosylation sites and at least one interchain disulfide bond, which can generate homodimers.

### 3.4 RNA blot analysis

Northern blot analysis of 4-1BB-L, shown in Fig. 4, demonstrated the presence of a single transcript of approximately 1.6 kb in various murine cell lines. This size correlated with the size of the isolated cDNA assuming a poly A tail of 2–300 nucleotides. Transcripts for 4-1BB-L were present in the bone marrow stromal cell line D11 and in bone marrow-derived macrophages, and transcript levels increased in both following induction. Transcripts were also detected in a thymic stromal cell line termed F4 and in a pre-B cell line Abl 1.1, but not in mature B cells. 4-1BB-L transcripts were also present in an induced T cell clone (7F9) but not in the uninduced cells. Many of these cells also expressed transcripts for 4-1BB, and its expression was co-regulated with that of 4-1BB-L (data not shown).

### 3.5 Chromosomal location of 4-1BB and 4-1BB-L genes

The mouse chromosomal locations of the 4-1bb receptor and its cognate ligand, 4-1bb-l, were determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F<sub>1</sub> × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1000 loci that are well distributed among all the autosomes as well as the X chromosome [24]. C57BL/6J and *M. spretus* DNA were digested with several enzymes and analyzed by Southern blot hybridization for informa-



**Figure 4.** RNA blot analysis of 4-1BB-L transcripts. Polyadenylated RNA (2 µg) or total RNA (5 µg; bone marrow-derived macrophages, B cells and 7F9) was electrophoresed through a formaldehyde-containing gel, blotted onto Hybond-N nylon membranes (Amersham Corporation), and probed with a labeled antisense murine 4-1BB-L RNA.

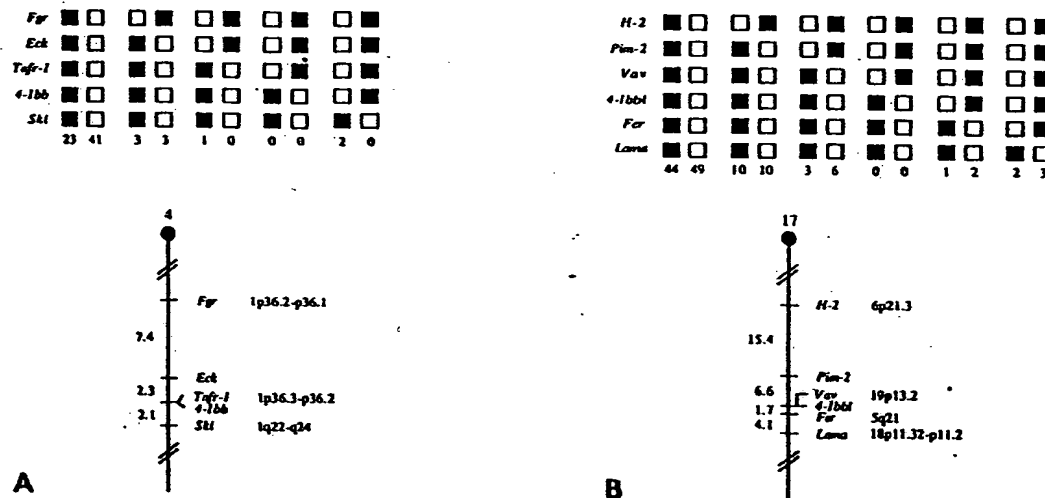
tive RFLP using probes for *4-1bb* and *4-1bb-l*. The 7.2, 6.6, 3.6 and 2.1 kb *M. spretus* BglI RFLP (see Sect. 2.6) were used to follow the segregation of the *4-1bb* locus in backcross mice. The mapping results indicated that *4-1bb* is located in the distal region of mouse chromosome 4 linked to *Fgr*, *Eck*, *Tnfr-1* (p80), and *Ski*. Although 73 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 5A), up to 190 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere-*Fgr*-6/81 - *Eck*-3/130 - *Tnfr-1*-0/179 - *4-1bb*-4/190 - *Ski*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM)  $\pm$  the standard error] are - *Fgr*-7.4  $\pm$  2.9 - *Eck*-2.3  $\pm$  1.3 - (*Tnfr-1*, *4-1bb*)-2.1  $\pm$  1.0 - *Ski*. No recombinants were detected between *Tnfr-1* and *4-1bb* in 179 animals typed in common suggesting that the two loci are within 1.7 cM of each other (upper 95% confidence limit).

A 4.6 kb *M. spretus* Hinc II RFLP (see Sect. 2.6) was used to follow the segregation of the *4-1bb-l* locus in backcross mice. The mapping results indicated that *4-1bb-l* located in the middle region of mouse chromosome 17 linked to *H-2*, *Pim-2*, *Vav*, *Fer* and *Lama*. Although 130 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 5B), up to 185 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combina-

tions for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci, and the most likely gene order are: centromere-*H-2*-25/162 - *Pim-2*-11/168 - *Vav*-0/185 - *4-1bb-l*-3/179 - *Fer*-7/171 - *Lama*. The recombinant frequencies [expressed as genetic distances in centiMorgans (cM)  $\pm$  the standard error] are - *H-2*-15.4  $\pm$  2.8 - *Pim-2*-6.6  $\pm$  1.9 - (*Vav*, *4-1bb-l*)-1.7  $\pm$  1.0 - *Fer*-4.1  $\pm$  1.5 - *Lama*. No recombinants were detected between *Vav* and *4-1bb-l* in 185 animals typed in common suggesting that the two loci are within 1.6 cM of each other (upper 95% confidence limit).

We have compared our interspecific maps of chromosomes 4 and 17 with composite mouse linkage maps that report the map location of many uncloned mouse mutations (compiled by M. T. Davisson, T. H. Roderick, A. L. Hillyard, and D. P. Doolittle and provided from GBASE, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). *4-1bb* and *4-1bb-l* mapped in regions of the composite maps that lack mouse mutations with a phenotype that might be expected for an alteration in these genes (data not shown).

Finally, the distal region of mouse chromosome 4 shares a region of homology with human chromosome 1 (Fig. 5A). The fact that *4-1bb* does not recombine with *Tnfr-1*, which has been placed on human 1p36.3-p36.2, suggests that *4-1bb* will also map to human 1, perhaps on the short arm.



**Figure 5.** (A) *4-1bb* maps in the distal region of mouse chromosome 4. *4-1bb* was placed on mouse chromosome 4 by interspecific backcross analysis. The segregation patterns of *4-1bb* and flanking genes in 73 backcross animals that were typed for all loci are shown at the top of the panel. For individual pairs of loci, more than 73 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J  $\times$  *M. spretus*)F<sub>1</sub> parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 4 linkage map showing the location of *4-1bb* in relation to linked genes is shown at the bottom of the panel. Recombination distances between loci in centiMorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci mapped in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD). (B) *4-1bb-l* maps in the middle region of mouse chromosome 17. The segregation patterns of *4-1bb-l* and flanking genes in the 130 interspecific backcross mice typed for all loci are shown at the top of panel B.



In contrast, the middle region of mouse chromosome 17 shares regions of homology with several human chromosomes including 6, 19, 5, and 18 (summarized in Fig. 5B). Thus, it is difficult to predict the human localization of 4-1bb-L. However, this ligand did not recombine with *Vav*, which has been positioned on human 19p13.2, and 4-1bb-L may reside on 19p as well.

### 3.6 Biological activities of 4-1BB-L

Previous studies have shown that T lymphocytes express 4-1BB [1, 40]. We have shown by Northern blot analysis that 4-1BB is also expressed in activated thymocytes (data not shown), suggesting that interaction of 4-1BB with its ligand may provide a signal for thymocyte growth and/or differentiation. To test this hypothesis, thymocytes from adult C57BL/6 mice were grown in the presence of IL-2 plus Con A and fixed CV-1/EBNA cells expressing recombinant 4-1BB-L, and the proliferative response assayed by thymidine incorporation. The results shown in Fig. 6A indicate that the fixed cells expressing 4-1BB-L induced proliferation of thymocytes in a dose-dependent fashion. By contrast, fixed CV-1/EBNA cells transfected with a control plasmid failed to increase proliferation above the levels observed in cells cultured in Con A and IL-2 alone. Soluble 4-1BB/Fc, but not soluble TNFR/Fc, inhibited the costimulatory effect of 4-1BB-L indicating the effect was specific.

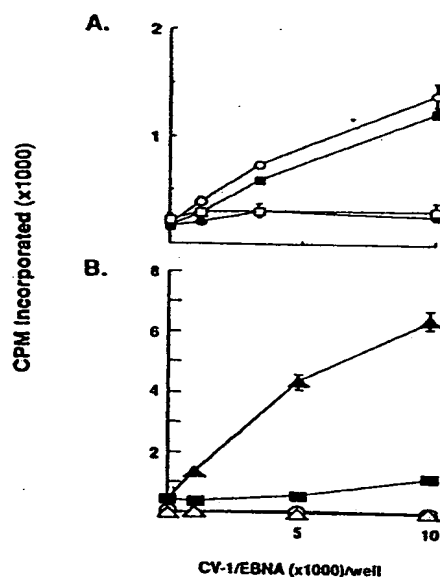


Figure 6. Biological effects of 4-1BB-L. (A) Purified murine thymocytes ( $3 \times 10^5$ /well) were cultured for 2 days in the presence of Con A and IL-2 with varying concentrations of fixed CV-1/EBNA cells transfected with vector alone (●) or with vector expressing 4-1BB-L (○). In addition to fixed cells expressing 4-1BB-L, cultures also included soluble 4-1BB/Fc (□) or soluble TNFR/Fc (■) at 10  $\mu$ g/ml. (B) Purified murine splenic T cells ( $2 \times 10^5$ /well) were cultured for 3 days with varying concentrations of fixed CV-1/EBNA cells transfected with vector alone (Δ) and in the presence of PHA (■) or with 4-1BB-L alone (○) and in the presence of PHA (▲).

Thymocyte costimulation by 4-1BB-L was also observed in cultures containing IL-2 alone, but not when cultured in the presence of Con A alone (data not shown).

To examine the effects of 4-1BB-L on mature T cells, purified murine splenic T cells were cultured in the presence or absence of a suboptimal dose of PHA (0.5%), with varying concentrations of fixed CV-1/EBNA cells transfected with empty vector or with vector expressing murine 4-1BB-L. Murine T cells were induced to proliferate in the presence of PHA, and this proliferation was augmented 3- to 15-fold in a dose-dependent manner by fixed CV-1/EBNA cells expressing 4-1BB-L but not by cells transfected with the empty vector (Fig. 6B). When T cells were cultured with 4-1BB-L alone in the absence of PHA, no proliferation over background was observed (Fig. 6B). The ability of 4-1BB-L to costimulate T cell proliferation was completely neutralized by addition of 4-1BB/Fc to the cultures but not by the addition of human IgG1 (data not shown).

### 4 Discussion

Here we report the isolation of a cDNA encoding a cognate for the T cell-induced antigen 4-1BB. Hydropathy analysis of the 4-1BB-L sequence, and binding studies with a soluble form of the ligand, have shown it to be a type II membrane protein with its carboxy-terminal domain expressed extracellularly. It was recently reported that 4-1BB also binds to extracellular matrix proteins, and it was suggested that these are the predominant physiological cognates [41]. However, this presumably occurs via interaction of O-linked carbohydrate on 4-1BB with matrix components. We propose, therefore, that the extracellular matrix instead may aid in the association of 4-1BB with the membrane-bound ligand described in this report.

Computer searches of the GenBank and EMBL sequence databases revealed that 4-1BB-L is unique. However, it does show similarity in the C-terminal portion of its extracellular domain with TNF and LT- $\alpha$ , as well as with CD40L and CD27L, all of which are ligands for other members of this same family of receptors. An alignment of the homologous region, which in TNF and LT- $\alpha$  has been shown to fold in the "jelly-roll"  $\beta$ -sandwich motif characteristic of viral capsid proteins [42-44], is shown in Fig. 7. Included in this alignment is the recently cloned LT- $\beta$  [14]. Though not a ligand for any of the known members of the receptor family, LT- $\beta$  associates with LT- $\alpha$  to form a heteromeric complex on the cell surface [14, 45]. In addition, we have recently isolated a cDNA which encodes a ligand for CD30 and found it is a type II membrane protein that also shows similarity in its C-terminal domain to this emerging family of ligands [46].

Among this family of ligands, 4-1BB-L exhibits the lowest level of sequence similarity in the homologous region: 14-16% identity with the various other family members. For comparison, human TNF and LT- $\alpha$  share 36% identity in this region, human TNF and CD40L share 26% identity, and human TNF and CD27L share 19% identity. Sequence similarity occurs primarily among the hydrophobic residues in  $\beta$ -strands B, C, D, and I (see Fig. 7), important for monomeric and trimeric structural integrity in TNF. This

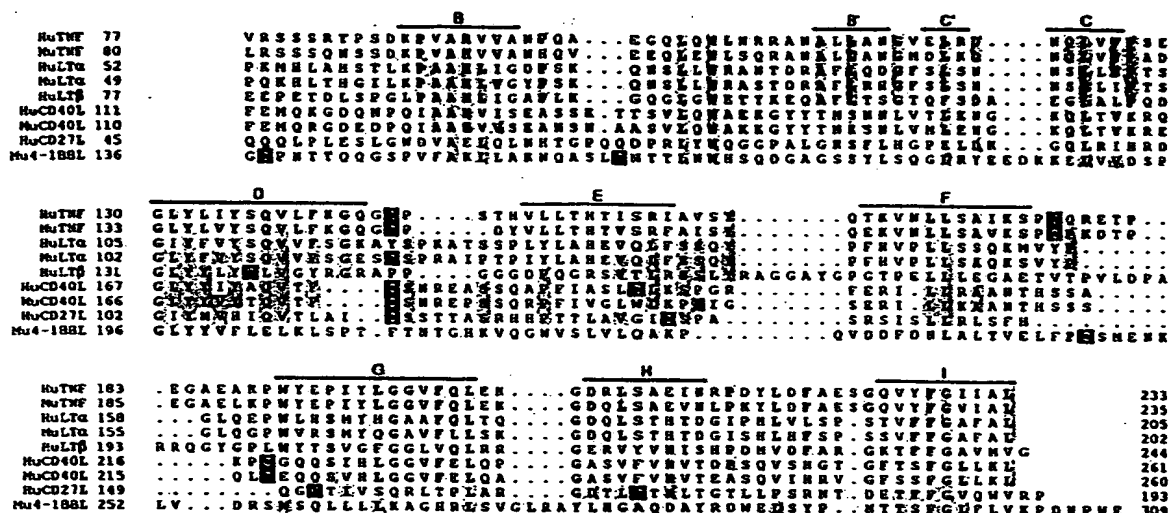


Figure 7. 4-1BB-L sequence homologies. The C-terminal domains of 4-1BB-L and other TNF ligand family members are aligned, beginning at the N terminus of mature TNF. Letters B-I indicated  $\beta$ -strands in the TNF crystal structure. Within the homologous region (strand B through C terminus) residues conserved in at least three family members (i.e., not counting conservation between homologous ligands in different species) are shaded gray. Cysteine residues are shaded black.

pattern of conservation indicates that 4-1BB-L has a tertiary structure very similar to that of TNF and LT- $\alpha$ , and is consistent with an oligomeric form for 4-1BB-L. The region between strands D and I is not well conserved and can only be loosely aligned, using secondary structure prediction techniques. There are also two conserved residues, Leu164 and Trp166, in the loop between  $\beta$ -strands B and B'. These residues just precede a segment of the loop, residues Gln169 through Ala172, that in TNF is important for activity [47]. The hydrophobic side-chains of Leu164 and Trp166 are buried and appear to anchor this important loop in place structurally.

The C-terminal residues of TNF and LT- $\alpha$  form  $\beta$ -strand I, an integral part of the tertiary fold. In 4-1BB-L there are seven extra C-terminal residues; these probably form a flexible tail that does not exist in the other family members. This putative tail is reminiscent of the N-terminal tails of soluble TNF and LT- $\alpha$  (9 and 25 residues, respectively) that modulate the biological activities of these cytokines [47].

Cysteine placement is diverse across the ligand family. Of the three cysteines in the extracellular domain of 4-1BB-L, the second and third cysteines, both in loops at the same end of the  $\beta$ -sandwich, are well-positioned to form a disulfide bond. The first cysteine (Cys137), 9 residues N-terminal to the homologous region, is thus probably the cysteine involved in the homodimer link.

The identified ligands for the TNF/NGF family of receptors exist in both membrane-bound and soluble forms. TNF, LT- $\beta$ , CD27L, CD30L and CD40L, like 4-1BB-L, are type II membrane proteins. Although LT- $\alpha$  has a signal sequence and is secreted from the cell, it can be found as a membrane-associated form via its interaction with the recently cloned LT- $\beta$  [14, 45]. The only other member of the

TNF/NGF receptor family of molecules which has identified ligands is the NGF receptor. These ligands, which include NGF and other members of the neurotrophin family, are secretory proteins that bind to the NGF receptor in association with the trk family of receptors [48]. NGF and the other neurotrophins are homologous to one another but show no sequence similarity to the TNF family of ligands. The structure of NGF has been determined [49], and, although it is an all- $\beta$  structure as is TNF, its topology is quite different.

Another common feature of the ligands for the family of TNF/NGF receptors is that they appear to be multimeric. TNF and LT- $\alpha$  have both been shown to be homotrimers. NGF exists as a 7S complex with the biologically active  $\beta$  subunit being a homodimer [50]. The 4-1BB-L clearly exists as a disulfide-linked homodimer (Fig. 3). The structure of CD40L is less clear though recent computer modeling studies suggest CD40L may exist as a non-covalent homo-trimer [51]. Multimeric forms of the ligands would be expected as the proposed mechanism of signaling by these molecules is via the cross-linking of their respective receptors.

Chromosomal mapping of 4-1BB demonstrated it was located very close to the gene for the larger form of the TNF receptor (p80) on the distal arm of mouse chromosome 4 [26]. Thus, human 4-1BB might be predicted to be on human chromosome 1 like the p80 TNF receptor [52]. The gene for CD30 is also closely linked to the p80 TNF receptor and 4-1BB, mapping to human chromosome 1p36 [53]. The p60 form of the TNF receptor and CD27 are also linked to each other, mapping to chromosome 12p13 [52, 54]. None of the other members of this family of receptors whose genes have been mapped are linked, with the Fas antigen mapping to human chromosome 10q24.1 [55], the murine

CD40 gene to the distal region of mouse chromosome 2 [56], and the NGF receptor to human chromosome 17q21-22 [57]. Nevertheless, the genomic organization of the members of this family of receptors further supports the hypothesis that they arose via gene duplication of an ancestral gene.

The family of homologous ligands also show some linkage with the genes for TNF, LT- $\alpha$ , and LT- $\beta$  all tightly linked in the major histocompatibility complex on mouse chromosome 17 [14]. The murine 4-1BB-L gene also maps to mouse chromosome 17, but considerably distal to the TNF and LT genes and likely resides on a different human chromosome. The other identified ligands of this family are unlinked, with the human CD27L gene on chromosome 19p13 [13], and the human CD40L on the X chromosome [58]. Analysis of the structures of the genes for these ligands should aid in determining if they also arose by gene duplication.

The induced expression of both 4-1BB and its ligand on activated T cells suggests that the interaction of these molecules may play a role in the T cell activation process. In support of this, it was recently shown that cross-linking of 4-1BB on anti-CD3 activated T cells with an mAb to 4-1BB resulted in an enhancement of T cell proliferation [59]. Here we have demonstrated that the interaction of 4-1BB with its ligand elicits the same response, acting as a proliferative co-stimulus on activated thymocytes and splenic T cells. This response is very similar to that generated by another member of the TNF family of molecules in that both CD27 and its ligand are expressed on activated T cells, and their interaction results in the increased proliferation of activated T cells [13]. Thus, recent evidence suggests that activated T cells express an emerging panel of cell membrane-bound molecules, the most carefully studied secondary signal for which is mediated by CD28 [60]. From data presented here it seems apparent that 4-1BB-L is another inducible T cell surface molecule that also contributes to T cell stimulation. It will be of interest to determine the precise role for each of these receptor-ligand interactions in an immune response and what unique function they each may play.

We thank Debra J. Gilbert for excellent technical assistance, John Sims, Linda Park and Steven Gillis for critical review of the manuscript, and Anne Bannister for assistance with the preparation of the manuscript.

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number L15435.

Received June 11, 1993; accepted July 21, 1993.

## 5 References

- 1 Kwon, B. S. and Weissman, S. M., *Proc. Natl. Acad. Sci. USA* 1989. 86: 1963.
- 2 Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D. and Goodwin, R. G., *Science* 1990. 248: 1019.
- 3 Loetscher, H., Pan, Y.-C. E., Lahm, H.-W., Gentz, R., Brockhaus, M., Tabuchi, H. and Lesslauer, W., *Cell* 1990. 61: 351.
- 4 Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H. W., Gatanaga, T., Granger, G. A., Lentz, R., Raab, H., Kohr, W. J. and Goeddel, D. V., *Cell* 1990. 61: 361.
- 5 Smith, C. A., Davis, T., Wignall, J. M., Din, W. S., Farrah, T., Upton, C., McFadden, G. and Goodwin, R. G., *Biochem. Biophys. Res. Commun.* 1991. 176: 335.
- 6 Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M. and Chao, M., *Cell* 1986. 47: 545.
- 7 Stamenkovic, I., Clark, E. A. and Seed, B., *EMBO J.* 1989. 8: 1403.
- 8 Mallet, S., Fossum, S. and Barclay, A. N., *EMBO J.* 1990. 9: 1063.
- 9 Camerini, D., Walz, G., Loeenen, W. A. M., Borst, J. and Seed, B., *J. Immunol.* 1991. 147: 3165.
- 10 Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S.-I., Sameshima, M., Hase, A., Seto, Y. and Nagata, S., *Cell* 1991. 66: 233.
- 11 Dürkop, H., Latza, U., Hummel, M., Eitelbach, F., Seed, B. and Stein, H., *Cell* 1992. 68: 421.
- 12 Armitage, R. J., Fanslow, W. C., Ströckbine, L., Sato, T. A., Clifford, K. N., Macduff, B. M., Anderson, D. M., Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., Clark, E. A., Smith, C. A., Grabstein, K. H., Cosman, D. and Spriggs, M. K., *Nature* 1992. 357: 80.
- 13 Goodwin, R. G., Alderson, M. R., Smith, C. A., Armitage, R. J., VandenBos, T., Jerzy, R., Tough, T. W., Schoenborn, M. A., Davis-Smith, T., Hennen, K., Falk, B., Cosman, D., Baker, E., Sutherland, G. R., Grabstein, K. H., Farrah, T., Giri, J. G. and Beckmann, M. P., *Cell* 1993. 73: 447.
- 14 Browning, J. L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E. P., Hession, C., O'Brien-Greco, B., Foley, S. F. and Ware, C. F., *Cell* 1993. 72: 847.
- 15 Sims, J. E., March, C. J., Cosman, D., Widmer, M. B., MacDonald, H. R., McMahon, C. J., Grubis, C. E., Wignall, J. M., Call, S. M., Friend, D., Alpert, A. R., Gillis, S. R., Urdal, D. L. and Dower, S. K., *Science* 1988. 241: 585.
- 16 Namen, A., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D., Schmierer, A., Mosley, B., March, C., Urdal, D., Gillis, S., Cosman, D. and Goodwin, R., *Nature* 1988. 333: 571.
- 17 McMahon, C. J., Slack, J. L., Mosley, B., Cosman, D., Lupton, S. D., Brunton, L. L., Grubin, C. E., Wignall, J. M., Jenkins, N. A., Brannan, C. I., Copeland, N. G., Huebner, K., Croce, C. M., Cannizzaro, L. A., Benjamin, D., Dower, S. K., Spriggs, M. K. and Sims, J. E., *EMBO J.* 1991. 10: 2821.
- 18 Urdal, D. L., Call, S. M., Jackson, J. J. and Dower, S. K., *J. Biol. Chem.* 1988. 263: 2870.
- 19 Larsen, A., Davis, T., Curtis, B. M., Gimpel, S., Sims, J. E., Cosman, D., Park, L., Sorensen, E., March, C. J. and Smith, C. A., *J. Exp. Med.* 1990. 172: 1559.
- 20 Dower, S. K., Kronheim, S., March, C. J., Hopp, T., Conlon, P. J., Gillis, S. and Urdal, D. L., *J. Exp. Med.* 1985. 162: 501.
- 21 Gearing, D. P., King, J. A., Gough, N. M. and Nicola, N. A., *EMBO J.* 1989. 8: 3667.
- 22 Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J., *Biochemistry* 1979. 18: 5294.
- 23 Cosman, D., Cerretti, D. P., Larsen, A., Park, L., March, C., Dower, S., Gillis, S. and Urdal, D., *Nature* 1984. 312: 768.
- 24 Copeland, N. G. and Jenkins, N. A., *Trends Genet.* 1991. 7: 113.
- 25 Jenkins, N. A., Copeland, N. G., Taylor, B. A. and Lee, B. K., *J. Virol.* 1982. 43: 26.
- 26 Goodwin, R. G., Anderson, D., Jerzy, R., Davis, T., Brannan, C. I., Copeland, N. G., Jenkins, N. A. and Smith, C. A., *Mol. Cell. Biol.* 1991. 11: 3020.
- 27 Lindberg, R. A. and Hunter, T., *Mol. Cell. Biol.* 1990. 10: 6316.
- 28 Siracusa, L. D., Jenkins, N. A. and Copeland, N. G., *Genetics* 1991. 127: 169.
- 29 Breuer, M. L., Cuypers, H. T. and Berns, A., *EMBO J.* 1989. 8: 743.
- 30 Katrav, S., Martin-Zanca, D. and Barbacid, M., *EMBO J.* 1989. 8: 2283.
- 31 Letwin, K., Yee, S.-P. and Pawson, T., *Oncogene* 1988. 3: 621.

- 32 Sasaki, M., Kleinman, H. K., Huber, H., Deutzmann, R. and Yamada, Y., *J. Biol. Chem.* 1988. 263: 16536.
- 33 Green, E. L., in (Eds.), *Genetics and Probability in Animal Breeding Experiments*, Oxford University Press, New York 1981, p. 77.
- 34 Fanslow, W. C., Clifford, K. N., Park, L. S., Rubin, A. S., Voice, R. F., Beckmann, M. P. and Widmer, M. B., *J. Immunol.* 1991. 147: 535.
- 35 Cerritini, J. C., Engers, H. D., MacDonald, H. R. and Bruener, K. T., *J. Exp. Med.* 1974. 140: 703.
- 36 Group, G. C., *Program Manual for the GCG Package*, Version 7, April 1991, 575 Science Drive, Madison, WI 53711.
- 37 Clark, S. P., *Comput. Appl. Biosci.* 1992. 8: 535.
- 38 Cohen, F. E., Abarbanel, R. M., Kunz, I. D. and Fletterick, R. J., *Biochemistry* 1986. 25: 266.
- 39 Mohler, K. M., Torrance, D. S., Smith, C. A., Goodwin, R. G., Stremler, K. E., Fung, V. P., Madani, H. and Widmer, M. B., *J. Immunol.* 1993. 151: 1548.
- 40 Kwon, B. S., Kestler, D. P., Eshhar, Z., Oh, K.-O. and Wakulchik, M., *Cell. Immunol.* 1989. 121: 414.
- 41 Chalupny, N. J., Peach, R., Hollenbaugh, D., Ledbetter, J. A., Farr, A. G. and Aruffo, A., *Proc. Natl. Acad. Sci. USA* 1992. 89: 10360.
- 42 Eck, M. J. and Sprang, S. R., *J. Biol. Chem.* 1989. 264: 17595.
- 43 Jones, E. Y., Stuart, D. I. and Walker, N. P., *Prog. Clin. Biol. Res.* 1990. 349: 321.
- 44 Eck, M. J., Ultsch, M., Rinderknecht, E., de Vos, A. M. and Sprang, S. R., *J. Biol. Chem.* 1992. 267: 2119.
- 45 Browning, J. L., Androléwicz, M. J. and Ware, C. F., *J. Immunol.* 1991. 147: 1230.
- 46 Smith, C. A., Gruss, H.-J., Davis, T., Anderson, D., Farrah, T., Baker, E., Sutherland, G. R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., Grabstein, K. H., Gliniak, B., McAlister, I. B., Fanslow, W., Alderson, M., Falk, B., Gimpel, S., Gillis, S., Din, W. S., Goodwin, R. G. and Armitage, R. J., *Cell* 1993. 73: 1349.
- 47 Goh, C. R. and Porter, A. G., *Protein Eng.* 1990. 4: 385.
- 48 Klein, R., Jing, S., Nanduri, V., O'Rourke, E. and Barbacid, M., *Cell* 1991. 65: 189.
- 49 McDonald, N. Q., Lapatto, R., Murray-Rust, J., Gunning, J., Wlodawer, A. and Blundell, T. L., *Nature* 1991. 354: 411.
- 50 Pintar, J. E., in Guraff, G. (Eds.), *Oncogenes, Genes and Growth Factors*, John Wiley and Sons, New York 1987, p. 103.
- 51 Peitsch, M. C. and Jongeneel, C. V., *Int. Immunol.* 1993. 5: 233.
- 52 Baker, E., Chen, L. Z., Smith, C. A., Callen, D. F., Goodwin, R. and Sutherland, G. R., *Cytogenet. Cell Genet.* 1991. 57: 117.
- 53 Fonatsch, C., Latza, U., Dürkop, H., Rieder, H. and Stein, H., *Genomics* 1992. 14: 825.
- 54 Loenen, W. A. M., Gravestein, L. A., Beumer, S., Melief, C. J. M., Hagemeijer, A. and Borst, J., *J. Immunol.* 1992. 149: 3937.
- 55 Fukunaga, R. W., Brannan, C. I., Itoh, N., Yonehara, S., Copeland, N. G., Jenkins, N. A. and Nagata, S., *J. Immunol.* 1992. 148: 1274.
- 56 Grimaldi, J. C., Torres, R., Kozak, C. A., Chang, R., Clark, E. A., Howard, M. and Cockayne, D. A., *J. Immunol.* 1992. 149: 3921.
- 57 Sehgal, A., Patil, N. and Chao, M., *Mol. Cell. Biol.* 1988. 8: 3160.
- 58 Allen, R. C., Armitage, R. J., Conley, M. E., Rosenblatt, H., Jenkins, N. A., Copeland, N. G., Bedell, M. A., Edelhoff, S., Distche, C. M., Simoneaux, D. K., Fanslow, W. C., Belmont, J. and Spriggs, M. K., *Science* 1993. 259: 990.
- 59 Pollok, K. E., Kim, Y.-J., Zhou, Z., Hurtado, J., Kim, K. K., Pickard, R. T. and Kwon, B. S., *J. Immunol.* 1993. 150: 771.
- 60 Linsley, P. S., Clark, E. A. and Ledbetter, J. A., *Proc. Natl. Acad. Sci. USA* 1990. 87: 5031.
- 61 Gallis, B., Prickett, K. S., Jackson, J., Slack, J., Schooley, K., Sims, J. E. and Dower, S. K., *J. Immunol.* 1989. 143: 3235.

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